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Mammalian Fatty Acid Synthase Activity From Crude Tissue Lysates Tracing [¹³C]-Substrates Using GC Mass Spectrometry

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Abstract

Fatty Acid Synthase (FASN, FAS; EC 2.3.1.85) is the sole mammalian enzyme to synthesize fatty acids *de novo* from acetyl and malonyl coenzyme A esters. A new method is described that directly quantifies uniformly labeled [¹³C]₁₆-palmitate by tracing [¹³C]₂-acetyl-CoA and [¹³C]₃-malonyl-CoA using an *in vitro* FASN assay. This method used GC-MS to detect [¹³C]₁₆-palmitate carboxylate anions (*m/z* 271) of pentafluorobenzyl derivatives and was highly sensitive at femtomole quantities. Uniformly incorporated [¹³C]₁₆-palmitate was the primary product of both recombinant and crude tissue lysate FASN. Quantification of FASN protein within crude tissue lysates assured equal FASN amounts, preserved steady state kinetics, and enabled calculation of FASN specific activity. FASN activity determined by [¹³C]₁₆-palmitate synthesis was consistent with values obtained from NADPH oxidation assays. Analysis of FASN activity from tissue extracts was not hampered by contaminating enzymes or pre-existing fatty acids. Crude mammary gland and liver lysates had significantly different activities at 82 and 65 nmoles minute⁻¹ mg⁻¹ respectively, suggesting tissue specific activity levels differ in a manner unrelated to FASN amount. GC-MS quantification of [¹³C]₁₆-palmitate synthesis permits sensitive evaluation of FASN activity from tissues of varied physiologic states, and of purified FASN activity in the presence of modifying proteins, enzymes, or drugs.

Keywords

Fatty Acid Synthase Activity; GC-Mass Spectrometry; Mammary Gland/Liver Lysates; [¹³C]-substrate incorporation; [¹³C]₁₆-Palmitic Acid

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Introduction

Animal Fatty Acid Synthase (FASN, FAS; EC 2.3.1.85) is expressed in most human tissues and is indispensable in the mouse as the FASN null mice are embryonic lethal [1; 2]. FASN is a complex enzyme that is absolutely essential for the cellular synthesis of short and medium chain fatty acids in mammals [3]. Mammalian FASN is a ≈ 270 kDa enzyme that contains seven enzymatic domains and harbors the complete pathway to synthesize palmitic acid *de novo* from acetyl and malonyl esters of coenzyme A as substrates (Figure 1). The products of FASN, free fatty acids, have multiple biological functions including lipid storage, phospholipid biosynthesis, both endocrine and nuclear hormone signaling ligands, and post-translational modification of proteins [4; 5; 6; 7; 8]. FASN abundance and *de novo* fatty acid synthesis activity are commonly de-regulated in a wide variety of human cancers and in metabolic diseases, underscoring the importance for the ability to study the enzyme [9; 10; 11]. Importantly, the enzymatic conversion of acetyl-CoA and malonyl-CoA substrates into palmitic acid can be recapitulated in the test tube, which has fostered elegant dissection of the mechanisms underlying FASN activity [3].

The majority of *in vitro* FASN activity assays can be grouped into two main categories: 1) assays that measure consumption/production of reaction components; and 2) assays that measure incorporation of heavy atom labeled substrates into the fatty acid products. Assays in the first group commonly quantify NADPH oxidation into NADP⁺ by monitoring changes in UV absorbance at 340 nm. Meticulous biochemistry in the 1990's determined the stereochemistry for the hydrogen atoms derived from NADPH, water, or malonyl-CoA during elongation of the fatty acyl chain to confirm that 14 molecules of NADPH are consumed for each palmitate synthesized [12; 13]. Researches have utilized this phenomenon to measure loss of absorption at 340 nM due to NADPH oxidation as a surrogate for FASN activity [14]. An alternative to monitoring NADPH consumption is to monitor the production of free coenzyme A (CoA) measured using fluorescent molecules that react with the free thiol of CoA, such as 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) [15; 16]. However, free CoA plays a critical role in the initial substrate sorting steps of the FASN reaction, and as a result, scavenging of CoA using covalent dyes such as CPM could adversely affect FASN catalysis [17]. In addition to CoA scavenging, thiol-reactive dyes have the potential to bind to FASN, and this interaction could alter catalysis. Moreover, use of thiol-reactive compounds to measure FASN activity in a crude tissue or cell lysate is likely to have decreased sensitivity due to dye interaction with molecules in the lysates. Despite these pitfalls, indirect assays for FASN activity remain popular and are used successfully largely because they use basic laboratory equipment, are easily scalable, and are therefore well suited for high throughput screens.

FASN activity assays in the second category measure the end product of the FASN: *de novo* synthesized, non-esterified, fatty acids. The majority of these assays rely upon incorporation of a radioactive precursor into synthesized fatty acids and quantification of reaction products by liquid scintillation counting [18]. Others have used non-radioactive tracers including D₂O or [¹³C]₁-malonyl CoA incorporation to measure palmitate synthesis with success [19; 20]. NADPH oxidation, radioactive tracers, and non-radioactive tracer methods have been problematic for a variety of reasons including the indirect measurement of palmitate synthesis, loss of information about fatty acid chain length, hazardous radioactive waste, isotope purity of substrates, deuterium isotope effects, and the decarboxylation of malonyl-CoA from crude tissue extracts to name a few. In this study, we describe a novel gas chromatography mass spectrometry (GC-MS) based FASN activity assay that uses high isotopic purity [¹³C]₂-acetyl-CoA and [¹³C]₃-malonyl-CoA reaction substrates to directly measure FASN product, *de novo* synthesized uniformly incorporated [¹³C]₁₆-palmitate. This method is well suited for small scale *in vitro* reactions with sensitivity at the femtomole

level, uses commercially available 99% isotope purity substrates and standards, and calculates FASN specific activity directly from crude extracts obviating the need for complicated FASN purifications.

Materials and Methods

Gas Chromatography Mass Spectrometry Fatty Acid Quantitation

Methods used in this study were adapted from Zarini et al. [21]. Sample analysis was performed by negative ion chemical ionization GC/MS using the Finnigan DSQ GC-MS system (Thermo Finnigan, Thousand Oaks, CA), with a ZB-1 column (15 m – 0.25 mm inner diameter 0.10 mm film thickness; Phenomenex). The GC was programmed to increase the temperature from 125 to 230°C at 20°C/min, 230 to 245°C at 5°C/min, and finally 245 to 300°C at 30°C/min, before being held at 310°C for 1min. The mass spectrometer was operated in the negative ion chemical ionization (NICI) mode using methane as reagent gas. Data were acquired in full scan mode to identify palmitic acid, [^{13}C]₁₆-palmitate (m/z 271) and the internal standard [^{13}C]₄-palmitate ion (m/z 259). Peak areas of the analyte or of the standard were measured, and the ratio of the area from the analyte derived ion to that from the internal standard was calculated. The ratios were then compared with the calibration curve (see below) for the analyte prepared from commercially available standards to quantify [^{13}C]₁₆-palmitate.

Palmitic Acid Calibration Curve

[^{13}C]₁₆-palmitate, 1,2,3,4-[^{13}C]₄-palmitate, and [D]₃₁-palmitate were purchased from Sigma Aldrich, St. Louis, MO with 99 atom % ^{13}C and 98 atom % D, respectively. Stock solutions for each compound were 1 mg/mL in 90% methanol. [^{13}C]₁₆-palmitate was the analyte and [^{13}C]₄-palmitate was the internal standard. The standard curve was prepared as a 5-fold serial dilution, and the analyte to standard ratios were measured in triplicate at seven different analyte amounts ranging from 460 picomoles to 150 femtomoles, with the internal standard held constant at 8 picomoles. Analyte to standard dilutions for generation of the standard curve made in 90% methanol, were acidified to pH 4.0, isooctane extracted, derivatized, and transferred to vials as described below (see *Sample Preparation for GC-MS*), with the exception that the derivatized pellet for the 460 picomole point was resuspended in 1.0 mL isooctane and the 150 femtomole point was resuspended in 25 μL isooctane.

Sample Preparation for GC-MS

HPLC grade reagents were purchase from Sigma Aldrich, St. Louis, MO. Following the acidification step, 8 picomoles of [^{13}C]₄-palmitate was added as the internal reference standard, which locked the analyte to standard ratio prior to fatty acid extraction. Fatty acids were extracted with 1.0 mL of isooctane; samples were vortexed vigorously for 10 seconds and allowed to stand for 5 min at room temperature, after which they were centrifuged for 1 minute at 300 x G. An 800 μL volume of the top organic phase was transferred to new glass tubes and taken to dryness using a Savant Speed Vac. Pellets were resuspended in 30 μL of 1% pentafluorobenzyl bromide in acetonitrile and 30 μL of 1% N,N-diisopropylethylamine dissolved in acetonitrile was added to initiate derivatization [21]. The derivatization reaction was allowed to proceed at room temperature for 30 minutes, after which the samples were dried using a Savant Speed Vac. The resulting pellets were resuspended in 100 μL (unless otherwise noted) of isooctane and transferred into vials for GC-MS.

Fatty Acid Synthase Activity Assay

Reactions were performed in a total volume of 200 μ L and FASN assay buffer consisted of 0.1 M potassium phosphate pH 6.6, 1.0 mM DTT, 1.0 mM EDTA, 1,2- ^{13}C ₂-acetyl-CoA at 99 atomic % ^{13}C , 1,2,3- ^{13}C ₃-malonyl-CoA at 99 atomic % ^{13}C , and β -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH) were purchased from Sigma Aldrich, St. Louis, MO. Reactions contained 2 μ g (representing 36.7 nM dimer concentration) of recombinant FASN or FASN isolated from tissues (see below). Recombinant FASN or FASN isolated from tissues was diluted to 0.2 μ g/ μ L in 250 mM potassium phosphate pH 7.0, 1.0 mM DTT, and 1.0 mM EDTA, and were then equilibrated to room temperature for 1.0 hour to restore FASN dimers. FASN assay buffer, 40 μ M ^{13}C ₂-acetyl-CoA, 180 μ M NADPH, and 10 μ L of diluted FASN (2 μ g total) were pre-incubated at 37° C for 2 minutes. The reactions were initiated by addition of 110 μ M ^{13}C ₃-malonyl-CoA and mixed. Following a 60 second reaction time, the samples were quenched with 2 volumes (500 μ L) of ice-cold methanol, acidified with 40 μ L of 1.0 M HCL to pH $\approx$ 3.5, and then gently vortexed. Cerulenin was purchased from Sigma Aldrich and dissolved to 5 mM in DMSO. In experiments with cerulenin, samples were incubated in the FASN assay buffer without substrates for 30 minutes at room temperature, alone, with 6 μ L DMSO (2% v/v), or with 1.0, 2.0, 10, 50 or 100 μ M cerulenin (each in 6 μ L DMSO). Following cerulenin incubation, assay was conducted as before. Samples were then prepared for GC-MS as described above.

Recombinant FAS

Recombinant rat FASN enzyme was kindly provided by Drs. S. Smith and A. Witkowski, (Children's Hospital Oakland Research Institute, Oakland CA). Enzyme concentration was measured using a Nanodrop spectrophotometer (Thermo Scientific), and concentration was estimated using a 280 nm extinction coefficient of 482,200 M⁻¹cm⁻¹ (dimer, without disulfides) according to Protein Calculator v3.3 (www.scripps.edu/~cdputnam/protcalc.html) using rat FASN primary sequence at www.UniProt.org (entry P12785). Details of the preparation of C-terminally FLAG tagged recombinant FASN can be found elsewhere [22]. Recombinant FASN protein was used to generate a standard curve to quantify the amount of FASN present in biological samples isolated from tissues. Triplicate dilutions ranging from 10 to 900 ng of FASN protein were run on SDS polyacrylamide gels, transferred to low fluorescence PVDF membrane (Millipore, Billerica, MA.), and incubated with 1:1000 FASN primary antibody from Santa Cruz Biotechnology (rabbit anti-human FASN, sc-20140, Santa Cruz, CA) which was generated against a 299 amino acid region in the C-terminus of human FASN (amino acids 2205–2504). The immunoblots were incubated in 1:15000 goat anti-rabbit IRDye 800CW secondary (LI-COR Biosciences, Omaha, NE.). Infrared immunoblots were scanned using the LiCor Odyssey system and data were analyzed using LiCor Image Studio 2.0 software (LI-COR Biosciences, Omaha, NE.). Amounts of endogenous FASN were calculated according to the following equation:

$$\left(\text{Regression Slope in } \frac{\text{ng FASN}}{\text{Signal Intensity}} \right) \times \left(\frac{\text{Signal Intensity}}{60 \text{ ug Cytosolic Protein}} \right) = \frac{\text{ng FASN}}{\text{ug Cytosolic Protein}}$$

Isolation of FASN from mouse Mammary Gland and Liver

Animals were maintained in the Center for Comparative Medicine; under protocols approved by the Institutional Animal Care and Use Committee of University of Colorado Denver. C57Bl/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and maintained on standard laboratory chow and standard day/night cycle. Mammary gland

epithelial cells were prepared as previously described, with the exception that PMSF was omitted from the process [23]. Liver tissues from the identical mice used for preparation of mammary epithelial cells were collected and immediately snap frozen on dry ice. Approximately 150–250 mg of tissue was homogenized in 1 mL of FASN lysis buffer [250mM sucrose, 20 mM HEPES pH 7.6, 2 mM MgCl₂, 1 mM DTT, 1 mM EDTA, 50 mM NaF, 5 mM NaVO₄, and 3x Roche complete EDTA-free protease inhibitors]. Homogenized samples were centrifuged at 3,500 x G for 10 minutes, supernatant was collected and cleared at 100,000 x G for 30 minutes by ultra-centrifugation. The protein concentrations present in the resulting cytosolic fraction were estimated using Pierce 660nM Protein Assay (ThermoFisher). A 60 µg amount of total cytosolic protein was loaded to denaturing SDS-PAGE and transferred to low fluorescence PVDF membrane (Millipore, Billerica, MA.). Immunoblotting of FAS was performed as above using anti-FAS primary antibody, the Licor goat anti-rabbit IRDye 800cw secondary, and scanned using the LiCor Odyssey system. The amount of FASN present in tissue lysates were quantified by comparison to a standard curve using purified recombinant FASN. The volume of cytosolic extract representing 2 µg of FASN protein levels was evaluated using the FASN activity assay as above.

Results

GC-MS Chromatograms and Mass Spectra of Fatty Acids

Gas chromatography resolved individual pentafluorobenzyl fatty acid esters of discrete chain lengths based on chromatography retention times. Pentafluorobenzyl bromine (PFB-Br) and diisopropylethylamine (DIEA) were used as the derivatization agents that generated fatty acid carboxylate anions under negative ion chemical ionization. The abundance of fatty acid carboxylate anions eluted from the column was detected by the mass spectrometer, which provided far superior signal to noise than other methods such as analysis of fatty acid methyl esters. Four palmitate isotopomers were purchased from a commercial vendor, including unlabeled palmitate, [¹³C]₄-palmitate, [¹³C]₁₆-palmitate, and [D]₃₁-palmitate, and an optimal internal reference standard was determined. These isotopomers of palmitic acid were mixed together, derivatized, and then the mixture was subjected to GC-MS analysis. Identical retention times for unlabeled and [¹³C]-labeled palmitate were observed in the extracted ion chromatogram (Figure 2A), but the retention time of the [D]₃₁-palmitate (*m/z* 286) standard was shifted 8.4 seconds earlier. The unique *m/z* values of each palmitic acid isotopomer was readily distinguished by the mass spectrometer for the unlabeled palmitate (*m/z* 255), [¹³C]₄-palmitate (*m/z* 259), [¹³C]₁₆-palmitate (*m/z* 271), and [D]₃₁-palmitate (*m/z* 286) carboxylate anions (Figure 2B). Also detected were the carboxylate anions of the natural 1.1% abundance of [¹³C] of the isotopomers for unlabeled palmitate (*m/z* 256), [¹³C]₄-palmitate (*m/z* 260), and [D]₃₁-palmitate (*m/z* 287). No natural 1.1% abundance of [¹³C] ion (e.g. *m/z* 272) was detected for [¹³C]₁₆-palmitate because all 16 carbons of palmitate were already [¹³C] labeled. Instead, a small fraction of [¹²C]-palmitate was detected (*m/z* 270) due to the 99 atom % [¹³C] isotopic purity from the commercial vendor. Additionally, a large percentage of the [D]₃₁-palmitate produced *m/z* 285, *m/z* 284, and *m/z* 283 ions, indicating that the 98 atom % D for this labeled palmitate was less than desirable. [¹³C]₄-palmitate was used as the internal standard and not [D]₃₁-palmitate because the retention time was identical to [¹³C]₁₆-palmitate and that [¹³C]₄-palmitate (*m/z* 259) differed by 12 daltons from the [¹³C]₁₆-palmitate (*m/z* 271) synthesized *in vitro* by FASN.

A standard curve for quantification was established using [¹³C]₁₆-palmitate as the analyte and [¹³C]₄-palmitate as the internal standard. Six different amounts of [¹³C]₁₆-palmitate that spanned almost four orders of magnitude (ranging 0.15 to 460 picomoles) were subjected to GC-MS analysis. The signal ratio of the analyte to the standard relative to the amount of analyte generated a standard regression curve (Figure 3A). The dilution series was

conducted in triplicate and had an R^2 correlation value of 0.9991 with a slope of 0.104 ± 0.00007 , and the inset shows linearity of the low end of the curve (0.15 to 3.7 picomoles of analyte). This method was highly sensitive and routinely capable of detecting $[^{13}\text{C}]_{16}$ -palmitate fatty acid carboxylate anions at and below 150 femtomoles. The relative abundance of the 460 picomole and the 150 femtomole $[^{13}\text{C}]_{16}$ -palmitate analyte (front peaks) to the internal $[^{13}\text{C}]_4$ -palmitate 8 picomole standard (back peaks) is demonstrated in the extracted ion chromatograms (Figure 3A and B). The strong, linear correlation value of the standard curve, combined with the sensitivity of the mass spectrometer using pentafluorobenzyl fatty acid derivatives enabled measurements with a broad dynamic range down to femtomole amounts of $[^{13}\text{C}]_{16}$ -palmitate.

FASN Activity Assay

Conditions of the *in vitro* assay were established using affinity purified recombinant rat FASN isolated from Sf9 insect cells infected with the full length FASN-encoding baculovirus expression vector. FASN activity assays have been well defined in the literature [18; 24] and were conducted with minor modifications according to Materials and Methods. FASN dimerization is required for complete enzyme activation and subsequent synthesis of palmitic acid, but dimer assembly is sensitive to temperatures below 20°C and solutions of low ionic strength (< 50 mM potassium phosphate buffer), such that FASN dimers slowly dissociate under either condition [25]. A critical aspect of either the $[^{13}\text{C}]_{16}$ -palmitate synthesis or NADPH oxidation methods for FASN activity was fully assembled FASN dimers. Recombinant FASN or tissue derived FASN was routinely incubated in 0.25 M potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM DTT, and 10% glycerol for one hour at room temperature to facilitate FASN dimer assembly. Without this process, only 20% of the fully reactivated FASN activity was observed (data not shown). The need to reactivate recombinant, purified, or crude tissue lysate FASN was underscored by the observation that FASN reactivation (dimer assembly) occurred during the reaction at 37°C, as the rate of $[^{13}\text{C}]_{16}$ -palmitate synthesis was parabolic up to two minutes into the reaction when the rate then became linear.

The primary product of recombinant FASN was $[^{13}\text{C}]_{16}$ -palmitate (m/z 271) when the reaction was supplied with $[^{13}\text{C}]_2$ -acetyl-CoA and $[^{13}\text{C}]_3$ -malonyl-CoA substrates (Figure 4A). The ion m/z 270 $[^{13}\text{C}]_{16}$ -palmitate was also routinely detected, which was due to the 99 atomic % $[^{13}\text{C}]$ of the two substrates. Also detected in the FASN activity assay was unlabeled palmitate (m/z 255) that originated from the stock of purified recombinant enzyme. Presence of m/z 255 unlabeled palmitate ion was verified by extracting fatty acids from the recombinant enzyme stock alone (data not shown). 50 μL volumes were sampled every 10 seconds for 60 seconds, and the reaction proceeded linearly and under steady state kinetics (Figure 4B). Working reaction conditions were determined for optimal steady state kinetics of FASN by varying substrate concentrations over a longer time series, and the best time frame and substrate concentrations that yielded the most optimal slope and linearity (R^2 correlation value) were selected. Later time points, such as 90 and 120 seconds, using 40 μM $[^{13}\text{C}]_2$ -acetyl-CoA, 110 μM $[^{13}\text{C}]_3$ -malonyl-CoA tended to plateau indicating that the reaction had deviated from steady state kinetics presumably due to high concentrations of free CoA that inhibits FASN catalysis [17]. The amount of $[^{13}\text{C}]_{16}$ -palmitate synthesized in the reaction was quantified relative to the analyte to standard ratio regression curve (Figure 3). FASN synthesized $[^{13}\text{C}]_{16}$ -palmitate at 101 nmoles $\text{min}^{-1} \text{mg}^{-1}$ (Figure 4B), and the increased m/z 271 $[^{13}\text{C}]_{16}$ -palmitate (front peak) relative to m/z 259 $[^{13}\text{C}]_4$ -palmitate standard (back peak) from 10 to 60 seconds (left and right insets, respectively) was represented in extracted ion chromatograms. The NADPH oxidation method was then compared to the direct quantification of $[^{13}\text{C}]_{16}$ -palmitate (Table 1). NADPH oxidation was monitored continuously via the spectrophotometric method and FASN oxidized overall 1544

nmoles NADPH min⁻¹ mg⁻¹ (+/- 137) compared to the [¹³C]₁₆-palmitate at 102 nmoles min⁻¹ mg⁻¹ (+/- 4.9) synthesized by FASN in that set of experiments. Because 14 molecules of NADPH are oxidized per molecule of [¹³C]₁₆-palmitate synthesized, 102 × 14 gives a converted NADPH oxidation of 1428 nmoles min⁻¹ mg⁻¹. Thus, the two methods differ by 7.5 % and are in reasonable agreement when measuring the activity of recombinant FASN.

Pentafluorobenzyl derivatives of [¹³C]₁₆-palmitate from commercial sources generated a carboxylate anion at *m/z* 271. Spectra from commercial [¹³C]₁₆-palmitate compared to spectra from recombinant FASN validated that [¹³C]₁₆-palmitate was a direct product of FASN catalysis. Additionally, exclusion of NADPH from the reaction resulted in no *m/z* 271 carboxylate anion from [¹³C]₁₆-palmitate and therefore no FASN catalysis occurred (Figure 5). [¹³C]₁₆-palmitate synthesis was evaluated in the presence of the ‘suicide’ FASN inhibitor cerulenin. Cerulenin is known to irreversibly inhibit the beta-ketoacyl synthase condensing enzyme domain of type I fatty acid synthases, and inhibitory concentrations are known to be directly proportional to FAS inhibition [26; 27]. The addition of DMSO (2% v/v), the solvent for cerulenin, did not alter the synthesis of [¹³C]₁₆-palmitate, and nearly the same reaction rate was observed compared to no cerulenin (Figure 5). Cerulenin was added at various concentrations to reactions, and reactions were carried out according to Materials and Methods. [¹³C]₁₆-palmitate synthesis was inhibited by 20% (*p* = 0.0018) with 1 μM cerulenin and suppressed 88% by the addition of 50 μM cerulenin at 12.8 (+/- 1.5) nmoles min⁻¹ mg⁻¹ relative to no cerulenin conditions at 104 nmoles min⁻¹ mg⁻¹ (Figure 5). Addition of 100 μM cerulenin resulted in near complete (98%) inhibition of [¹³C]₁₆-palmitate synthesis (32%–98% samples, *p* = 0.0005). By using [¹³C]-labeled acetyl-CoA and malonyl-CoA incorporation to directly measure [¹³C]₁₆-palmitate synthesis, this method was capable of investigating enzyme inhibition *in vitro* with sensitivity enough to detect very subtle changes in catalysis at lower than reported drug concentrations. A significant 20% inhibition was observed (*p* = 0.002) at 1.0 μM cerulenin. These data demonstrate a specific and sensitive method to directly quantify FASN catalyzed synthesis of [¹³C]₁₆-palmitate.

FASN activity assay from tissue extracts

Of great interest is to evaluate the FASN activity directly from crude lysates of a wide variety of tissues and cell lines under normal and pathological conditions. A critical aspect when using crude cell or tissue extracts is the amount of ‘contaminating enzymes’ within the cytosolic fraction [28]. Contaminating enzyme activity was generally avoided using complicated FASN purification methods such as anion exchange chromatography [29]. Added to the difficulty of evaluating FASN activity from crude lysates was that FASN protein amounts could not be quantified. Calculation of FASN amount from crude lysates is necessary for several reasons: to assure that the amount of enzyme from each sample is equivalent, to be certain that the levels of FASN in the *in vitro* assay preserve steady state kinetics, and to calculate of the specific activity based on milligrams of FASN. Crude cytosolic lysate FASN from mammary gland and liver tissues was quantified using an immunoblot approach relative to a standard regression curve generated with recombinant rat FASN (Figure 6A). The anti-FASN antibody is a rabbit polyclonal generated against a 299 amino acid region (2205–2504) at the C terminus of human FASN. There is 95% sequence identity (283/299 amino acids) between human and mouse FASN in this region, a 94% identity (280/299 amino acids) between human and rat FASN in this region, and a 97% identity between rat and mouse FASN (296/304 amino acids), suggesting that the cross reactivity between rat and mouse epitopes is robust. Immunoblots were quantified using the Licor Odyssey instrument, and each standard curve dilution point was evaluated in triplicate. A regression line was generated with a slope of 0.0001 and an R² correlation value of

0.99885. Next, crude cytosolic fractions were prepared from mammary gland epithelial cells (MEC) and matched livers of the same animals as described in Materials and Methods and amounts of crude cytosolic lysate FASN were calculated using the recombinant FASN regression curve. When standardized to 60 μg of total cytosolic fraction protein, crude lysate FASN was quantified using the signal intensity values from all immuno-reactive bands shown (Figure 6B). FASN amounts were consistent from the MECs but variable from the liver. β -tubulin indicated that cytosolic protein in each well, but was not used for any quantification or normalization purposes. Crude lysate FASN from MECs and liver FASN was estimated at 38.6 ($\pm$ 1.9) and 19.8 ($\pm$ 9.7) nanograms per microgram of cytosolic protein, respectively. Using this strategy to quantify endogenous FASN in each sample (table in 6B), a known FASN amount was added to the following activity assays.

FASN from crude cytosolic extracts of both tissues synthesized $[^{13}\text{C}]_{16}$ -palmitate (m/z 271) as the primary reaction product (Figure 7). A large quantity of pre-existing unlabeled palmitate derived from the cytosolic fractions of the lysates was detected (m/z 255 ion, shown) as were unsaturated fatty acids 18:1 m/z 281 and 18:2 m/z 279 that did not originate from FASN catalysis in the assay (data not shown). Detection of pre-existing fatty acids in the lysates emphasized the importance of supplying uniformly $[^{13}\text{C}]$ -labeled substrates in the crude FASN assay reaction, as variable levels of unlabeled palmitate would confound palmitate detection using unlabeled substrates. The ion m/z 270 resulted from the 99 atom % $[^{13}\text{C}]$ isotopic purity of the substrates as was observed from the recombinant FASN assays. The specific activity of FASN from the crude cytosolic MEC and the liver lysates was calculated (Figure 7B). Equalized amounts (2 μg) of MEC and Liver FASN synthesized $[^{13}\text{C}]_{16}$ -palmitate at 82 and 65 $\text{nmole min}^{-1} \text{mg}^{-1}$ FASN, respectively. The specific activity of FASN from in vivo samples was 20–35% less than that observed for recombinant FASN. Although the amount of FASN from the crude cytosolic extracts was equal, the mammary gland FASN had significantly higher specific activity (1.26 fold; $p = 0.02$) compared to liver FASN, a trend that was observed in three independent experiments. This observation suggested that the 1.26 fold difference might be due to factors other than overall enzyme abundance. Finally, addition of 100 μM cerulenin inhibited FASN catalysis by greater than 93% (Figure 7B), which validated that presence of the $[^{13}\text{C}]_{16}$ -palmitate m/z 271 carboxylate anion in reaction using crude lysates was due to FASN.

Discussion

FASN has been studied by biochemists for decades to elucidate enzyme mechanism, reaction kinetics, and to determine the requirements for enzyme catalysis. Here, a new method is described to directly quantify $[^{13}\text{C}]_{16}$ -palmitate synthesis that utilizes incorporation of high purity, non-radioactive heavy atom $[^{13}\text{C}]_2$ -acetyl-CoA and $[^{13}\text{C}]_3$ -malonyl-CoA substrates (Figure 1). This method detected carboxylate anions of $[^{13}\text{C}]_{16}$ -palmitate pentafluorobenzyl derivatives using GC-MS. GC-MS simultaneously measured both the $[^{13}\text{C}]_{16}$ -palmitate analyte and the $[^{13}\text{C}]_4$ -palmitate internal standard at the same retention time to provide greater precision than non-internal standards [30]. The finding that the primary product of FASN is saturated palmitate was validated using this method by comparing the m/z 271 ion of $[^{13}\text{C}]_{16}$ -palmitate to purchased $[^{13}\text{C}]_{16}$ -palmitate. GC-MS directly quantifies *de novo* fatty acid synthesis *in vitro* and the specific activity for both recombinant FASN and tissue-derived FASN was determined. This new method integrates use of $[^{13}\text{C}]_2$ -acetyl-CoA and $[^{13}\text{C}]_3$ -malonyl-CoA heavy atom tracers with the power of GC-MS to provide a very sensitive assay that detects FASN catalyzed *de novo* synthesized fatty acids at the femtomole level. Most importantly, this method is not confounded by the presence of unlabeled palmitate that often contaminates solvents and blanks since it measures $[^{13}\text{C}]_{16}$ -palmitate synthesis from uniformly $[^{13}\text{C}]$ -labeled acetyl- and malonyl-coenzymeA esters.

The direct quantification of [^{13}C] $_{16}$ -palmitate was compared to the NADPH oxidation method using recombinant FASN and identical reaction conditions according to Materials and Methods. The values observed in Table 1 were consistent with previous reports of FASN activity calculated using the NADPH oxidation method that indicated a broad range of values between 1500–2000 nmoles $\text{min}^{-1} \text{mg}^{-1}$ [24; 29; 31; 32]. In our hands, the NADPH oxidation method gave 1544 nmoles $\text{min}^{-1} \text{mg}^{-1}$. The direct [^{13}C] $_{16}$ -palmitate quantification method was consistent with, but slightly lower than, reported results at approximately 1428 nMol NADPH oxidized $\text{minute}^{-1} \text{mg}^{-1}$ (converted by multiplying 102 nmoles $\text{min}^{-1} \text{mg}^{-1}$ times 14). The [^{13}C] $_{16}$ -palmitate quantification value is slightly lower presumably because we monitored the abundance of only the [^{13}C] $_{16}$ -palmitate carboxylate anion at m/z 271. To that end, other FASN products are synthesized in the reaction including myristic and stearic acids, and short acyl ‘by-products’ of FASN such as butyryl (4:0) or crotonyl (4:1) CoA esters [33]. With respect to palmitate synthesis, these additional FASN products all consume NADPH over the course of the reaction. Since oxidation of NADPH was used to calculate FASN catalysis, production of these minor products alters calculations of FASN activity from NADPH oxidation during continuous 340 nM monitoring of the aqueous reaction mixture using the spectrophotometric method. This observation suggested the difference in calculation of specific activity could be a result of the methods used rather than due to variations in catalysis of recombinant FASN itself.

Previous methods to quantify fatty acids typically used radioactive materials and liquid scintillation counting to measure fatty acid synthesis. While this procedure successfully measures the products of FASN catalysis, critical information regarding fatty acid chain length cannot be determined using this approach. Conventional gas chromatography is useful to discriminate among fatty acids of varied chain length [4]; however, heavy atom tracer information would be lost using standard flame ionization. Radiolabeled gas chromatography has been used to quantify fatty acids of various chain lengths by retention time and it retains radioactive tracer content, however quantification of each fatty acid is accomplished using reference standards that are unrelated to the fatty acid of interest [34; 35]. Radiolabeled HPLC methods also exist to resolve and detect discrete fatty acid chain lengths using flow through scintillation detectors [33; 36]. While the sensitivity of radio assays is quite good, the accuracy is often poor [20]. Both liquid scintillation counting and radio chromatography techniques utilize radioactive materials that are often expensive, require special handling, and cause difficulty for waste disposal due to the generation of hazardous, mixed wastes. This method directly quantified FASN activity using [^{13}C] $_2$ -acetyl-CoA and [^{13}C] $_3$ -malonyl-CoA incorporation to avoid the undesirable issues with radio isotope methods.

Two methods were reported for either deuterium or [^{13}C] $_1$ -malonyl CoA incorporation into labeled palmitate using a similar *in vitro* FASN activity assay and GC-MS [19; 20]. Monitoring deuterium incorporation was not desirable due to isotope effects of deuterium in the FASN assay [13; 19]. Alternatively, Ohashi, et al., compared the NADPH method with [^{13}C] $_1$ -malonyl CoA incorporation into [^{13}C]-labeled de novo fatty acids synthesized by column-purified FASN and FASN from crude cytosolic extracts. A major issue with this study was the isotopic purity of the [^{13}C] $_1$ -malonyl CoA used in the FASN assay: [^{13}C] $_1$ -malonyl CoA was synthesized and purified in the lab at 92 atom % [^{13}C] isotopic purity with the label at the central methylene carbon of malonyl-CoA. Using lower purity [^{13}C] $_1$ -malonyl CoA, Ohashi et al. detected [^{13}C] $_6$ -, [^{13}C] $_7$ -, and [^{13}C] $_8$ -incorporated palmitate requiring summation of these ions. Conversely, commercial sources exist currently to provide 99 atom % [^{13}C] isotopic purity of [^{13}C] $_2$ -acetyl-CoA and [^{13}C] $_3$ -malonyl-CoA substrates, which enables synthesis of completely incorporated [^{13}C] $_{16}$ -palmitate (m/z 271) and only minor amounts of the [^{13}C] $_{15}$ carboxylate anion m/z 270 (~ 10%). Another improvement from the method reported by Ohashi and colleagues, who measured fatty acid

methyl esters and McLafferty rearrangement ion m/z 74, is the quantitation of carboxylate anions of non-fragmented fatty acids. PFB-Br/DIEA derivatization of [^{13}C] $_{16}$ -palmitate generated carboxylate anions of fatty acids that were robustly detected by the mass spectrometer resulting in an excellent signal to noise ratio. The derivatization strategy for this method was sensitive in the low femtomole range, which was far superior to either the NADPH oxidation method or the quantitation of fatty acid methyl esters. Finally, the previous [^{13}C] $_1$ -malonyl CoA used nMolar concentrations of substrates over a 30 minute reaction, which did not measure fatty acid synthesis reaction under steady state conditions from either column-purified FASN or from crude extracts.

Researchers have routinely isolated FASN from mammary tissues and liver, and some have purified FASN from tumors and tumor cell lines to investigate enzyme activity [18; 37; 38]. However, calculating FASN concentrations from crude lysates of biological samples has been difficult. Addition of known FASN amounts to the reaction is essential to assure that the analysis is conducted under steady state conditions. Herein, is an immunoblotting approach to calculate the amount of FASN eliminating the need for enzyme purification that typically abolishes protein-protein interactions that may be important in understanding the cell-type specific biology. To that end, crude tissue lysates are known to contain 'interfering enzymes' that could consume NADPH, decarboxylate malonyl-CoA, or alter acetyl-CoA levels [20; 28; 39]. For example, the presence of interfering enzymes might affect calculations of FASN activity via NADPH oxidation if NADPH is oxidized by factors other than FASN. Additionally, decarboxylation of malonyl CoA by malonyl-CoA decarboxylase in the crude lysate results in acetyl CoA, which would alter the levels of malonyl-CoA that is needed for acyl elongation by FASN. Conversely, this method supplied micromolar concentrations of [^{13}C] $_2$ -acetyl-CoA, [^{13}C] $_3$ -malonyl-CoA, and NADPH, which insulated FASN catalysis against interfering enzymes present in crude lysates. The method outlined is therefore suitable to conduct FASN assays using tissue derived crude cytosolic fractions.

FASN protein from crude *in vivo* extracts was quantified to enable calculation of the specific activity based on milligrams of FASN added to the reaction. The mammary gland samples contained several minor bands detected in the immunoblot but not present in the liver samples. It is not clear if these minor products represent a catalytically functional FASN or if they are break down products of non-functional FASN. The specific activity of FASN from the crude extracts was 20–35% less than that observed for recombinant FASN. It is possible that the immunoblotting approach used to quantify mouse FASN in crude cytosolic extracts, relative to a rat FASN regression curve, introduced error into the calculation of specific activity for the mouse enzyme. However, the epitope of the anti-FASN antibody for mouse and rat was highly similar (97% identical), and is unlikely to account for the 20–35% difference in activity. This means that the trend observed between mouse tissue extracts would not be altered, even though the "absolute" amount of total FASN could be variable. The difference between mouse crude extract FASN and the affinity purified rat FASN could be species specific; however, column purified FASN from species as divergent as rat and chicken had nearly identical activities at 1500–2000 versus 1600 nMol NADPH oxidized min $^{-1}$ mg $^{-1}$, respectively [24; 29]. On the other hand, the 20–35% difference was consistent with the observations that compared recombinant FASN with tissue derived FASN, where the tissue derived FASN had lower activity than the purified recombinant FASN [29]. We speculate that the difference in activity between the mouse and the rat synthases is most likely due to the type of FASN preparation used, crude cytosolic extract versus affinity purified recombinant FASN.

Importantly, calculating the specific activity of FASN in mouse tissues based on the immunoblotting quantification of FASN made it possible to discern if factors other than FASN amount influence the enzyme's activity under steady state conditions. The novel

observation that the specific activity of FASN from lactating mammary epithelial cells is significantly higher (1.26 fold; $p = 0.02$) when compared with FASN from liver, suggested that FASN activity may be modified in a tissue specific manner. It is tempting to speculate that the observed increase between the MECs and liver specific activity of FASN resulted from the presence of mammary specific factors that complex with FASN. Alternatively, this increase may be due to the presence of tissue specific fatty acid binding proteins. Binding of *de novo* synthesized fatty acids to binding proteins could enhance catalysis by relieving product inhibition of FASN. Alternatively, protein activators of FASN, such as 4'-phosphopantetheine transferase [40], might exist at different amounts in the two tissues thereby increasing pantetheinyl activation of the mammary gland FASN. In the end, it is clear that this method can be directly applied to assays with recombinant FASN, column-purified FASN, or to partially enriched FASN present in a cell or tissue cytosolic lysate.

In conclusion, we have described a new method that evaluates FASN activity based on the detection and quantitative measurement of enzyme product formation - *de novo* synthesized [^{13}C] $_{16}$ -palmitate using GC-MS. This method can be applied to tissues from all mammalian species, and it provides the opportunity to directly compare tissue preparations because FASN amounts are determined. FASN specific activity is reliably measured in tissues under different metabolic conditions or various physiological and diseased states, and [^{13}C] $_{16}$ -palmitate synthesis can now be precisely measured from crude extracts without complication from unlabeled fatty acids present in the lysates. FASN is generally seen as an indicator of aggressive tumors and poor patient outcome [9; 11] and therefore this assay could be useful to demonstrate a direct link between FASN activity and disease prognosis. Moreover, this method can be used to investigate the effects of FASN modifying proteins, inhibitory peptides, and small molecule inhibitors. Finally, important kinetic information will likely be obtained about FASN catalysis by direct quantitative measurement enzyme product. Sensitive quantitation of FASN catalysis will add to the understanding of this complex multi-component process, which is remarkably contained entirely within the single enzyme fatty acid synthase.

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Abbreviations

CoA	coenzyme A
DIEA	diisopropylethylamine
FASN	fatty acid synthase
GC-MS	gas chromatography mass spectrometry
MEC	mammary gland epithelial cells
NADPH	β -nicotinamide adenine dinucleotide 2'-phosphate
PFB	pentafluorobenzyl bromide

References

1. Chirala SS, Chang H, Matzuk M, Abu-Elheiga L, Mao J, Mahon K, Finegold M, Wakil SJ. Fatty acid synthesis is essential in embryonic development: fatty acid synthase null mutants and most of the heterozygotes die in utero. *Proc Natl Acad Sci U S A*. 2003; 100:6358–63. [PubMed: 12738878]

2. Jayakumar A, Tai MH, Huang WY, al-Feel W, Hsu M, Abu-Elheiga L, Chirala SS, Wakil SJ. Human fatty acid synthase: properties and molecular cloning. *Proc Natl Acad Sci U S A*. 1995; 92:8695–9. [PubMed: 7567999]
3. Smith S, Witkowski A, Joshi AK. Structural and functional organization of the animal fatty acid synthase. *Prog Lipid Res*. 2003; 42:289–317. [PubMed: 12689621]
4. Rudolph MC, Monks J, Burns V, Phistry M, Mariani R, Foote MR, Bauman DE, Anderson SM, Neville MC. Sterol Regulatory Element Binding Protein (SREBP-1) and Dietary Lipid Regulation of Fatty Acid Synthesis in the Mammary Epithelium. *Am J Physiol Endocrinol Metab*. 2010
5. Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, Lehmann JM. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc Natl Acad Sci U S A*. 1997; 94:4318–23. [PubMed: 9113987]
6. Ross J, Najjar AM, Sankaranarayananpillai M, Tong WP, Kaluarachchi K, Ronen SM. Fatty acid synthase inhibition results in a magnetic resonance-detectable drop in phosphocholine. *Mol Cancer Ther*. 2008; 7:2556–65. [PubMed: 18723500]
7. Rioux V, Legrand P. Saturated fatty acids: simple molecular structures with complex cellular functions. *Curr Opin Clin Nutr Metab Care*. 2007; 10:752–8. [PubMed: 18089958]
8. Cao H, Gerhold K, Mayers JR, Wiest MM, Watkins SM, Hotamisligil GS. Identification of a lipokine, a lipid hormone linking adipose tissue to systemic metabolism. *Cell*. 2008; 134:933–44. [PubMed: 18805087]
9. Menendez JA, Lupu R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nat Rev Cancer*. 2007; 7:763–77. [PubMed: 17882277]
10. Menendez JA. Fine-tuning the lipogenic/lipolytic balance to optimize the metabolic requirements of cancer cell growth: molecular mechanisms and therapeutic perspectives. *Biochim Biophys Acta*. 2010; 1801:381–91. [PubMed: 19782152]
11. Kuhajda FP. Fatty-acid synthase and human cancer: new perspectives on its role in tumor biology. *Nutrition*. 2000; 16:202–8. [PubMed: 10705076]
12. Anderson VE, Hammes GG. Stereochemistry of the reactions catalyzed by chicken liver fatty acid synthase. *Biochemistry*. 1984; 23:2088–94. [PubMed: 6722137]
13. Seyama Y, Kasama T, Yamakawa T, Kawaguchi A, Saito K. Origin of hydrogen atoms in the fatty acids synthesized with yeast fatty acid synthetase. *J Biochem*. 1977; 82:1325–9. [PubMed: 338601]
14. Horecker BL, Kornberg A. The extinction coefficients of the reduced band of pyridine nucleotides. *J Biol Chem*. 1948; 175:385–90. [PubMed: 18873313]
15. Chung CC, Ohwaki K, Schneeweis JE, Stec E, Varnerin JP, Goudreau PN, Chang A, Cassaday J, Yang L, Yamakawa T, Kornienko O, Hodder P, Inglese J, Ferrer M, Strulovici B, Kusunoki J, Tota MR, Takagi T. A fluorescence-based thiol quantification assay for ultra-high-throughput screening for inhibitors of coenzyme A production. *Assay Drug Dev Technol*. 2008; 6:361–74. [PubMed: 18452391]
16. Cerne D, Zitnik IP, Sok M. Increased fatty acid synthase activity in non-small cell lung cancer tissue is a weaker predictor of shorter patient survival than increased lipoprotein lipase activity. *Arch Med Res*. 2010; 41:405–9. [PubMed: 21044743]
17. Soulie JM, Sheplock GJ, Tian WX, Hsu RY. Transient kinetic studies of fatty acid synthetase. A kinetic self-editing mechanism for the loading of acetyl and malonyl residues and the role of coenzyme A. *J Biol Chem*. 1984; 259:134–40. [PubMed: 6706923]
18. Roncari DA. Fatty acid synthase from human liver. *Methods Enzymol*. 1981; 71(Pt C):73–9. [PubMed: 7278680]
19. Seyama Y, Kawaguchi A, Okuda S, Yamakawa T. New assay method for fatty acid synthetase with mass fragmentography. *J Biochem*. 1978; 84:1309–14. [PubMed: 730755]
20. Ohashi K, Otsuka H, Seyama Y. Assay of fatty acid synthetase by mass fragmentography using [¹³C]malonyl-CoA. *J Biochem*. 1985; 97:867–75. [PubMed: 4019438]
21. Zarini S, Gijon MA, Folco G, Murphy RC. Effect of arachidonic acid reacylation on leukotriene biosynthesis in human neutrophils stimulated with granulocyte-macrophage colony-stimulating

- factor and formyl-methionyl-leucyl-phenylalanine. *J Biol Chem.* 2006; 281:10134–42. [PubMed: 16495221]
22. Joshi AK, Rangan VS, Smith S. Differential affinity labeling of the two subunits of the homodimeric animal fatty acid synthase allows isolation of heterodimers consisting of subunits that have been independently modified. *J Biol Chem.* 1998; 273:4937–43. [PubMed: 9478938]
 23. Rudolph MC, Wellberg EA, Anderson SM. Adipose-depleted mammary epithelial cells and organoids. *J Mammary Gland Biol Neoplasia.* 2009; 14:381–6. [PubMed: 19953310]
 24. Cox BG, Hammes GG. Steady-state kinetic study of fatty acid synthase from chicken liver. *Proc Natl Acad Sci U S A.* 1983; 80:4233–7. [PubMed: 6576333]
 25. Smith S, Abraham S. Fatty acid synthetase from lactating rat mammary gland. 3. Dissociation and reassociation. *J Biol Chem.* 1971; 246:6428–35. [PubMed: 5167249]
 26. Omura S. The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. *Bacteriol Rev.* 1976; 40:681–97. [PubMed: 791237]
 27. Moche M, Schneider G, Edwards P, Dehesh K, Lindqvist Y. Structure of the complex between the antibiotic cerulenin and its target, beta-ketoacyl-acyl carrier protein synthase. *J Biol Chem.* 1999; 274:6031–4. [PubMed: 10037680]
 28. Bressler R, Wakil S. The Study of Fatty Acid Synthesis IX. THE CONVERSION OF MALONYL COENZYME A TO LONG CHAIN FATTY ACIDS. *Journal of Biological Chemistry.* 1961; 236:1643–1651.
 29. Joshi AK, Smith S. Construction of a cDNA encoding the multifunctional animal fatty acid synthase and expression in *Spodoptera frugiperda* cells using baculoviral vectors. *Biochem J.* 1993; 296(Pt 1):143–9. [PubMed: 8250834]
 30. Murphy RC, Gaskell SJ. New applications of mass spectrometry in lipid analysis. *J Biol Chem.* 2011; 286:25427–33. [PubMed: 21632539]
 31. Smith S, Abraham S. Fatty acid synthase from lactating rat mammary gland. *Methods Enzymol.* 1975; 35:65–74. [PubMed: 235708]
 32. Singh N, Wakil SJ, Stoops JK. On the question of half- or full-site reactivity of animal fatty acid synthetase. *J Biol Chem.* 1984; 259:3605–11. [PubMed: 6706971]
 33. Witkowski A, Joshi AK, Smith S. Characterization of the beta-carbon processing reactions of the mammalian cytosolic fatty acid synthase: role of the central core. *Biochemistry.* 2004; 43:10458–66. [PubMed: 15301544]
 34. Agradi E, Libertini L, Smith S. Specific modification of fatty acid synthetase from lactating rat mammary gland by chymotrypsin and trypsin. *Biochem Biophys Res Commun.* 1976; 68:894–900. [PubMed: 1259738]
 35. Libertini LJ, Smith S. Purification and properties of a thioesterase from lactating rat mammary gland which modifies the product specificity of fatty acid synthetase. *J Biol Chem.* 1978; 253:1393–401. [PubMed: 627544]
 36. Naggert J, Narasimhan ML, DeVeaux L, Cho H, Randhawa ZI, Cronan JE Jr, Green BN, Smith S. Cloning, sequencing, and characterization of *Escherichia coli* thioesterase II. *J Biol Chem.* 1991; 266:11044–50. [PubMed: 1645722]
 37. Kuhajda FP, Jenner K, Wood FD, Hennigar RA, Jacobs LB, Dick JD, Pasternack GR. Fatty acid synthesis: a potential selective target for antineoplastic therapy. *Proc Natl Acad Sci U S A.* 1994; 91:6379–83. [PubMed: 8022791]
 38. Ahmad PM, Feltman DS, Ahmad F. Studies on acetyl-CoA carboxylase and fatty acid synthase from rat mammary gland and mammary tumours. *Biochem J.* 1982; 208:443–52. [PubMed: 6130760]
 39. Wang X, Stanley WC, Darrow CJ, Brunengraber H, Kasumov T. Assay of the activity of malonyl-coenzyme A decarboxylase by gas chromatography-mass spectrometry. *Anal Biochem.* 2007; 363:169–74. [PubMed: 17316539]
 40. Joshi AK, Zhang L, Rangan VS, Smith S. Cloning, expression, and characterization of a human 4'-phosphopantetheinyl transferase with broad substrate specificity. *J Biol Chem.* 2003; 278:33142–9. [PubMed: 12815048]

Figure 1.

Reaction equation for the activity of Fatty Acid Synthase showing conversion of $[^{13}\text{C}]_2$ acetyl-CoA and $[^{13}\text{C}]_3$ malonyl-CoA substrates, NADPH, and protons into 16 carbon saturated $[^{13}\text{C}]_{16}$ -palmitic acid plus byproducts of enzyme catalysis.

Figure 2.

Extracted ion chromatogram and spectrum of commercially available isotopomers of palmitate (16:0) mixed together, extracted, derivatized and injected into the GC-MS. A) Resolution of pentafluorobenzyl fatty acid esters by chromatographic retention time. [¹³C]₄- and [¹³C]₁₆-palmitate co-eluted at 6.54 minutes with the unlabeled palmitate, but [D]₃₁-palmitate eluted from the column 0.14 minutes (8.4 seconds) earlier than the [¹³C]-palmitate molecules. B) Extracted ion spectrum from retention time 6.40 to 6.7 for carboxylate anions of palmitate isotopomers at m/z 255 (unlabeled), m/z 259 ([¹³C]₄-), m/z 271 ([¹³C]₁₆-), m/z 286 ([D]₃₁-).

Figure 3.

Panel A is the serial dilution regression curve of the $[^{13}\text{C}]_{16}$ -palmitate analyte to $[^{13}\text{C}]_4$ -palmitate standard ratio relative to the amount of analyte. The analyte amount varied from 0.15 to 460 picomoles and the standard was held at 8 picomoles. The dilution series was performed in triplicate with a slope of 0.104 and correlation value of 0.9991. Inset shows range from 0 to 1.0 ng of analyte. B) The extracted ion chromatograms for the 460:8 pmoles point (left) and the 0.15:8 pmoles point (right) for the $[^{13}\text{C}]_{16}$ -palmitate analyte (front peaks) and the $[^{13}\text{C}]_4$ -palmitate standard (back peaks). The sensitivity of this assay was 0.15 pmoles of $[^{13}\text{C}]_{16}$ -palmitate analyte.

Figure 4.

A) Extracted ion spectrum for $[^{13}\text{C}]_{16}$ -palmitate (m/z 271), $[^{13}\text{C}]_4$ -palmitate (m/z 259) standard, and unlabeled palmitate (m/z 255) from the recombinant FASN assay. The primary product is $[^{13}\text{C}]_{16}$ palmitate when supplying $[^{13}\text{C}]_2$ -acetyl-CoA and $[^{13}\text{C}]_3$ -malonyl-CoA substrates in the reaction. B) Recombinant FASN activity over 1 minute monitoring the $[^{13}\text{C}]_{16}$ -palmitate carboxylate anion (m/z 271). The reaction proceeds linearly demonstrating steady state kinetics and that recombinant FASN synthesized 101 nmoles $[^{13}\text{C}]_{16}$ -palmitate $\text{min}^{-1}\text{mg}^{-1}$. The abundance of $[^{13}\text{C}]_{16}$ -palmitate (front peak) relative to the $[^{13}\text{C}]_4$ -palmitate standard (back peak) in extracted ion chromatograms for the 10 and 60 second points increased over time (insets).

Figure 5.

Recombinant FASN activity assay performed according to conditions in Materials and Methods. Samples were incubated with and without increasing concentrations of cerulenin. The [¹³C]₁₆-palmitate carboxylate anion m/z 271 was monitored and quantified relative to the regression curve in 3A. Specific activity of recombinant FASN without cerulenin synthesized [¹³C]₁₆-palmitate at 104 nmoles min⁻¹mg⁻¹, and [¹³C]₁₆-palmitate synthesis was significantly inhibited by 20% ($p = 0.0018$) with 1 μ M cerulenin up to 98% with 100 μ M cerulenin (32%–98% samples, $p = 0.0005$). DMSO (2% v/v) had no effect on the reaction and the [¹³C]₁₆-palmitate carboxylate anion m/z 271 was not detected in the absence of NADPH.

Figure 6.

A) Regression curve for the serial dilution series using known amounts of recombinant FASN protein in micrograms. The dilution was performed in triplicate with a correlation value of 0.99885. The Slope of the dilution series was used to calculate the amount of FASN isolated from tissue extracts. B) Calculation of FASN quantity in ng/ μ g of cytosolic extract from mammary gland epithelium and from liver tissues. Values in the table are calculated from the signal intensity values quantified in the immunoblot for all immuno-reactive bands shown. These signal intensity values are relative to recombinant FASN regression curve in (A) and are calculated according to the equation in Materials and Methods. L denotes the marker ladder for the immunoblot.

A

Figure 7.

A) Extracted ion spectrum of the FASN activity assay using tissue derived FASN. The primary reaction product is uniformly incorporated [¹³C]₁₆-palmitate (*m/z* 271). Unlabeled palmitate (*m/z* 255) was detected in the spectrum from the crude cytosolic extract and [¹³C]₄-palmitate (*m/z* 259) is the internal standard. B) FASN activity assay using the volume of crude cytosolic extracts that corresponded to 2 μg total FASN from mammary epithelial cells and livers of the same mice. A 1.26 fold (*p* = 0.02) significant decrease in specific activity was observed by liver FASN for [¹³C]₁₆-palmitate synthesis. Reactions were conducted according to Materials and Methods without and with 100 uM cerulenin, inhibiting FASN activity greater than 93%. Data plotted were quantified by monitoring [¹³C]₁₄-myristic *m/z* 241, [¹³C]₁₆-palmitate (*m/z* 271), and [¹³C]₁₈-stearate *m/z* 301 ions.

Table 1
Comparison of NADPH oxidation with direct [¹³C]₁₆-palmitate quantitation

[¹³C]₁₆-palmitate quantification method was compared to the NADPH oxidation method using recombinant FASN and identical reaction conditions outlined in Materials and Methods. Oxidation of NADPH was continuously monitored on two different runs, and numbers are expressed as nmoles NADPH oxidized min⁻¹mg⁻¹. In two separate experiments, the [¹³C]₁₆-palmitate carboxylate anion m/z 271 was monitored and quantified relative to the regression curve in Figure 3. Specific activity of recombinant FASN using NADPH method was 1544 nmoles min⁻¹mg⁻¹ and FASN synthesized [¹³C]₁₆-palmitate at 102 nmoles min⁻¹mg⁻¹. NADPH oxidation was converted to nmoles of palmitate by dividing 1544 by 14 that gave 110 nmoles min⁻¹mg⁻¹, since 14 molecules of NADPH are oxidized during the synthesis of one palmitate molecule.

	Sample 1	Sample 2	Sample 3	Sample 4	Average
NADPH Run 1	1267.2	1459.2	1546.0	1612.8	1469 (+/- 77)
NADPH Run 2	1632.0	1536.0	1728.0	1584.0	1616 (+/- 100)
Overall Average	1544 (+/- 137)				
Converted	110.0				
[¹³ C] ₁₆ -Palmitate 1	98.4	101.4	96.8	109.4	101 (+/- 5.6)
[¹³ C] ₁₆ -Palmitate 2	108.8	98.5	105.2	100.2	103 (+/- 5.22)
Overall Average	102 (+/- 4.9)				